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"Self-replicating vector for DNA immunization against HIV"
(Itsereplikoituva vektori DNA-immunisaatiota varten HIV:tä vastaan)

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Self-replicating vector for DNA immunization against HIV

Field of the invention

The invention is directed to a self-replicating recombinant vector useful in DNA immunization against HIV. The invention is also directed to a vaccine comprising said vector, a method for preparing the vector, and a host cell comprising it. The invention further relates to the use of said vector for the manufacture of a vaccine against HIV and to a method of treating or preventing HIV.

Background of the invention

The interaction of vertebrates and thus also human beings with pathogenic microbes, such as bacteria, fungi and viruses is regulated by the capacity of the vertebrate organism to mount an immune response towards the invading microbe. This reaction is based on the capacity of immune system to distinguish between self and non-self; in normal situation immune response tolerates the own structures, cells and antigenic molecules of the organism while attacks foreign antigens, expressed by the invading microbes. When a vertebrate, such as man, is infected with microbes, the immune response helps in clearing the infection by killing microbes or cells infected with microbes or by preventing the spread of infection through the action of neutralizing antibodies. Secondly, and more importantly, immune response once elicited to an invading organism has an inborn mechanism of memory and thus an individual who has once experienced infection with a particular microbe is often immune and can not be infected the second time.

This immunological memory, caused in natural situation by infection is the basis for vaccines that mimic natural infection in many ways. An ideal vaccine will cause no or only slight symptoms in the vaccinated individuals but still result in induction of immunological memory with a capability to mount a strong preventive immune responsive in case the vaccine is encountered with the microbe in question.

The design of a vaccine against a particulate microbe is dependent on the mechanism by which organism in a natural infection may clear this particular organism and prevent subsequent infections. There are several ways how immune response can elicit its favorable function. Firstly, antibodies synthesized and secreted by the B lymphocytes can bind to microbe and through complement-mediated lysis destroy it. Secondly, neutralizing

antibodies can prevent a spreading of infection by inhibiting with the binding of the microbe to its target cell. Thirdly, antibodies in conjunction with complement activation can destroy infected cells, and, finally, specific cytotoxic T lymphocytes (CTL) can kill and destroy cells infected with the microbe. All these mechanisms have been thought to be involved in the immune response elicited by the human immunodeficiency virus (HIV) type 1 and 2 (HIV-1 and HIV-2). However, the immune response in HIV-infected individuals is usually characterized by a strong antibody response and less effective or even lacking T lymphocyte response (Gerstott, J. et al. Scand. J. Immunol. 22(5):463-470, 1985; Re, M.C. et al. J.Clinical Pathol. 42(5):282-283, 1989). This may be the reason why individuals, once infected with HIV developed a chronic infection despite the strong antibody-mediated immune response.

Preventive immune response towards viral infection in general can be mediated by the four types of immune response described above, but it is known that the CTL response, capable of killing viral infected cells is most effective. This is the reason why generally speaking live attenuated viral vaccines have proven to be most effective. In fact, the first vaccine developed by Jenner more than 200 years ago, the live vaccinia virus that can prevent the infection by the small-pox virus is an example of this principle. When an individual is vaccinated with a live attenuated viral vaccine, host cells are infected, viral proteins are synthesized. Some of the viral protein molecules are used to produce virus particles while others are proteolytically cleaved to small peptides that bind to the major histocompatibility (MHC) antigens (in man HLA class I and II) and are presented to T lymphocytes on the surface of infected cells. Subsequently, T lymphocytes carrying a proper T cell receptor (TCR) will recognize the foreign peptide in association with HLA and either give help to B cells for antibody production (helper/inducer T cells; Th) or destroy the infected cell (cytotoxic T lymphocyte; CTL).

In spite of a decade of efforts, an effective vaccine against human immunodeficiency virus (HIV) infection and AIDS is still lacking. Most earlier efforts have concentrated on obtaining sterilizing immunity with neutralizing antibodies against the outer envelope glycoprotein of HIV, gp120/gp160. Phase I/II studies with gp160/gp120 demonstrate, however, the neutralization against laboratory strains only but failure to neutralize field isolates.

In contrast, experiments with attenuated virus have been successful in the simian immunodeficiency (SIV) model. SIV deleted in the NEF or REV

gene behaves as an attenuated virus and protects the vaccinated animals against disease development but not against infection by the wild-type challenge virus. With a REV- defective virus, the only immunological correlate with protection was cell-mediated immune (CMI) response against SIV regulatory proteins NEF and TAT. An important observation in this experiment was, that vaccinated animals could even clear the infection caused by the wild-type challenge virus. It has recently been reported that also HIV-infected patients may occasionally clear an overt infection. The only relevant correlation to protection in these animal and/or human studies seems to be a cell mediated immune response towards HIV.

This type of immune response is generally not obtained with protein immunization. In terms of HIV, live attenuated vaccines could be effective to prevent infection but they are theoretically dangerous for several reasons. A recently described method, genetic immunization (synonyms: nucleic acid immunization, DNA immunization) has several of the advantages of live attenuated vaccines but not their potentially harmful adverse effects. A DNA vaccine, in a form of eukaryotic expression vector that carries the gene for one or a few of the viral proteins can induce the synthesis of the viral protein, once the DNA vector is transfected into host cell. Viral proteins synthesized in the target cell will then be processed by proteolytic enzymes; the formed peptides will be bound to MHC/HLA molecules and presented on the surface of transfected cells. This will cause a CTL-mediated immunological memory that in case the individual is subsequently infected with the virulent wild-type virus will be effective in killing viral infected cells immediately upon infection and thus preventing the infection. Direct intramuscular or intradermal injection of cDNA in an eukaryotic expression plasmid has been shown to induce an immune response (Wolff et al. Science 246:1465-1468, 1990). The expression of foreign antigens by such means results predominantly in helper T-cell subset 1 (TH1) type immune responses, with strong cytotoxic T-lymphocyte (CTL) response and, occasionally, also high- titer antibody response (Wang, B. et al, PNAS 90: 4156-4160. 1993; Wang et al. Ann. NY Acad. Sci 772: 186-197, 1995; Haynes et al. AIDS Res. Hum. Retroviruses 10 (2):43-45, 1994). Moreover, using viral nucleoprotein antigen of influenza A, antigen-specific CTL and protection has been reported (Ulmer. et al. Science 259, 1745-1749, 1993). Furthermore, protection against infection using DNA immunization has been obtained for mycoplasma in mice (Barry et al.

Nature 377(6550):632-635, 1995; Lai et al. DNA Cell. Biol. 14(7):643-651, 1995) and for human papillomavirus in a rabbit model (Donnelly et al. J. Inf. Dis. 173(2):314-320, 1996). DNA immunization has also been used to induce antitumor immunity mediated by cytotoxic lymphocytes (Bohm et al. Cancer Immunol. Immunother. 44(4):230-238, 1997).

DNA immunization has several advantages in comparison to live attenuated viral vaccines. As no infectious virus is formed, the viral genes induced to the host organism stay only in those cells that are originally transfected and no symptoms of virus infection occurs. In question of HIV, the major theoretical harmful effect for a live attenuated virus would be reversion, by mutations to a virulent wild-type virus. Furthermore, with DNA immunization only those viral genes, or parts of thereof that are known to be effective in inducing preventive immune response can be used.

For an effective CTL response, it would be important that the cytotoxic T-cells would destroy the infected cells before structural proteins are formed and prior to the release of mature viral particles. Therefore, immune response towards the early proteins in viral cycle would be beneficial. The replication of HIV is regulated by its own regulatory genes and proteins. The HIV genome encodes three nonstructural regulatory proteins (NEF, TAT, REV) which are indispensable for the replication of the virus in vivo. REV is a transporter of genomic RNA into the cytoplasm, TAT upregulates viral transcription and NEF provides replication in resting cells. Experiments with SIV indicate that viruses lacking the function of one of these regulatory genes may not be able to induce disease because of insufficient viral replication. These three proteins are expressed transiently and in small quantities during the first hours of the viral infectious cycle (Ranki et al. Arch.Virol. 139:365-378, 1994). Only a small proportion of HIV-infected individuals shows humoral and/or cellular response to these proteins, and the response correlates with a favorable clinical course.

CTL responses against NEF, TAT and REV have been extensively studied. NEF-specific CTL and Th (T helper cell) responses correlate with a favorable clinical prognosis. With a REV defective SIV-vaccine, immune responses to NEF and TAT were protective. Th and CTL epitopes in TAT and REV proteins, which are recognized by HIV-1 infected individuals and which show a clinical correlation, have been identified (Blazevic et al. J AIDS 6:881-890, 1993; Blazevic et al. AIDS Res. Hum. Retroviruses 11:1335-1341, 1995).

Taken together, these results indicate that for protection against disease a moderate replication of virus (attenuated growth) in combination with specific immune responses against the regulatory proteins involved in support of virus replication may be necessary.

Several eukaryotic expression vectors can be used in DNA immunization but their efficacy varies. Some of the parameters that regulate the efficacy of a given expression vector in inducing the immune response are unknown but obviously high level of expression of the antigenic protein would be advantageous. The time period that the vector, introduced to the cell can express the foreign antigenic viral protein may also be of importance. Finally, expression vectors that induce certain level of cell injury may also be advantageous as it is known that tissue destruction will amplify immune response through several biologically active molecules, such as cytokines, lymphokines and chemokines, secreted by the cell expressing the antigenic protein. This is probably one further reason why live attenuated virus that causes a certain level of tissue and cell destruction is so effective in inducing immunity, and thus a DNA vector that in this respect mimics live attenuated vaccine would be advantageous.

Nonspecific factors such as cytokines and lymphokines may also regulate the viral replication and immune responses in HIV-1 infection. The role of the helper cell Th1/Th2 balance, reflected by production of lymphokines specific for the two helper T-cell populations, has been demonstrated by Clerici and Shearer (Immunology Today 14(3):107-111, 1993) and others. Soluble factors, produced by CD8 cells and capable in suppressing the viral production by HIV-1 infected CD4 cells, were recently identified as RANTES, MIP1-α and MIP1-β (Cocchi et al. Science 270(5243):1811-1815, 1995). It is possible, that cytokines whose production is either increased or decreased in HIV-1 infection will regulate viral transcription.

Previous studies on DNA immunization using the gene encoding the HIV regulatory protein NEF have demonstrated T-cell proliferative responses (Hinkula et al. Vaccine 15 (8):874-878, 1997 and Hinkula et al. J. Virol. Jul, 71(7):5528-5539, 1997). However, it is the CTL response that has a positive effect correlation with a favorable clinical course.

One of the main objects of the present invention is therefore to provide a DNA immunization vaccine encoding an HIV regulatory protein, the vaccine being capable of eliciting a CTL response against HIV infected cells in

the early phase of the infectious cycle, before new mature infectious viral particles are released.

Another object of the invention is to provide a vaccine, which further elicits a humoral response against HIV.

A further object of the invention is to provide an HIV vaccine, which is safe to use, because it does not expose the recipient to the structural genes or proteins of HIV.

Another object of the present invention is to provide a self-replicating vector that causes a prolonged and high level of HIV regulatory protein expression and a certain degree of cell destruction, which will further stimulate the immune response.

Still another object of the invention is to provide a self-replicating recombinant vector expressing HIV regulatory proteins, which vector confers long-term stable maintenance and a high copy number in transfected cells including mammalian cells.

A further object of the invention is to provide a host cell comprising said vector.

Yet another object of the invention is to provide a method for preparing the above-mentioned self-replicating vector.

The present invention further provides a method of treating or preventing HIV.

Still another object of the invention is the use of said vector for the manufacture of a DNA immunization vaccine against HIV.

Summary of the Invention

The objects of the present invention can be achieved by incorporating a heterologous nucleotide sequence encoding the HIV regulatory protein NEF, REV or TAT or an immunologically active fragment thereof into a vector comprising a papilloma virus E1 gene and E2 gene, a minimal origin of replication of a papilloma virus and a minichromosomal maintenance element of a papilloma virus.

In other words the invention is directed to a self-replicating recombinant vector comprising papilloma virus nucleotide sequences consisting essentially of

(i) a papilloma E1 gene and E2 gene,

(ii) a minimal origin of replication of a papilloma virus

(iii) a minichromosomal maintenance element of a papilloma virus, and

a heterologous nucleotide sequence encoding the HIV regulatory protein NEF, REV or TAT or an immunologically active fragment thereof.

The invention further provides a vaccine for DNA immunization against HIV comprising said vector, the use of said vector for the manufacture of a vaccine against HIV, and a method of treating or preventing HIV comprising administering to a person in need thereof an effective amount of the self-replicating vector and expressing the NEF, REV or TAT protein or an immunologically active fragment thereof in said person.

The invention still provides a method for preparing a self-replicating recombinant vector, said method comprising

A) inserting a heterologous nucleotide sequence encoding the HIV regulatory protein NEF, REV or TAT or an immunologically active fragment thereof into a vector comprising papilloma virus nucleotide sequences consisting essentially of

- (i) a papilloma E1 gene and E2 gene,
- (ii) a minimal origin of replication of a papilloma virus, and
- (iii) a minichromosomal maintenance element of a papilloma virus, and

B) transforming a host cell with the resulting self-replicating recombinant vector,

C) culturing the host cell, and

D) recovering said vector.

The invention also provides a host cell comprising said vector.

Brief description of the Drawings

Figure 1A shows the shuttle vector pUE83.

Figure 1B shows the shuttle vector pNP177.

Figure 2 shows the pBNtkREV plasmid of the invention.

Figure 3 shows the pBNsrαTAT plasmid of the invention.

Figure 4 shows the pBNsrαNEF plasmid of the invention.

Figure 5 shows the NEF expression in COS-7 cells transfected with pBNsrαNEF. The Western blot samples are taken 72 h post transfection and visualized with ECL.

Figure 6 demonstrates anti-NEF antibodies in sera of mice immunized with pBNsrαNEF as detected in Western blot. Samples 1 - 4 were

taken 2 weeks post last immunization and samples 5 - 8 were taken 4 weeks post last immunization.

Figure 7 shows CTL responses in mice immunized with the pBNsrαNEF vector. Figure 7A shows CTL responses, expressed as % specific lysis of the target cells, in the four mice tested two weeks after the last immunization. Figure 7B shows the values at four weeks after the last immunization. Specific lysis > 4 % is considered positive.

Detailed description of the invention

According to the invention the heterologous HIV nucleotide sequence is inserted into a vector comprising a papilloma virus E1 gene and E2 gene, a minimal origin of replication of a papilloma virus (MO), and a minichromosomal maintenance element of a papilloma virus (MME). This E1/E2/MO/MME comprising vector is hereinafter called pBN, and it has been described in detail in WO 97/24451, which is incorporated by reference. Said patent publication is based on the discovery that DNA replication in papilloma viruses from the MO per se is not sufficient for stable long-term persistence, but in addition another viral sequence MME is required and that the best results are obtained when the vector further comprises the E1 and E2 genes of the papilloma virus.

'Papilloma virus' as used herein means any member of the papilloma virus family. Preferably the papilloma virus used in the invention is bovine papilloma virus (BPV) or human papilloma virus (HPV).

'E1' and 'E2' are regulatory proteins of papilloma viruses, which replicate via MO and which are necessary for replication.

'Minimal origin of replication' (MO) is a minimal sequence of a papilloma virus which is necessary for initiation of DNA synthesis.

'Minichromosomal maintenance element (MME) refers to a region of the papilloma viral genome to which viral or human proteins essential for papilloma viral replication bind. MME is essential for stable episomal maintenance of the papilloma viral MO in a host. Preferably MME comprises multiple binding sites for the transcriptional activator protein E2.

'Self-replicating vector' as used in the present application means a vector plasmid capable of autonomous replication in a eukaryotic host cell.

'Heterologous' means foreign. For example with respect to the vectors of the invention a heterologous nucleotide sequence means a non-papilloma sequence.

'Immunologically active fragment' means a fragment capable of eliciting an immunological response in a recipient.

'Papilloma virus nucleotide sequences consisting essentially of" means that the vector comprises the papilloma nucleotide sequences which are necessary and sufficient for long-term vector persistence and replication. This means for example that superfluous sequences like all papilloma-encoded oncogenic sequences have been deleted from the pBN vectors used in the present invention.

In addition to the E1 and E2 genes, MO, MME and the NEF, REV or TAT gene, the vectors of the invention comprise promotors for the encoded proteins as well as additional regulatory sequences, poly-adenylation sequences and introns. Preferably the vectors also include a bacterial host cell origin of replication and one or more genes for selectable markers for the preparation of the vector DNA in a bacterial host cell.

An essential feature of the pBN vectors is that they are not host cell specific. This is because the expression of the E1 and E2 proteins is controlled by promotors which are non-native i.e. heterologous. Said promotors are either functional in a broad range of mammalian cells or tissues or are cell- or tissue-specific.

In the vectors of the present invention the E1 gene is preferably under the control of the sr-α promotor or the thymidine kinase promotor (tk) and the E2 gene is preferably under the control of the LTR gag promotor. The NEF, REV or TAT gene can be under the control of a CMV promotor or an RSV LTR promotor. The vector can further comprise an SV40 early promotor to induce the expression of the gene for antibiotic selection (neomycin or kanamycin).

The host cell origin of replication in the vectors of the invention is preferably pUC ORI and the selective markers used are e.g. kanamycin and/or neomycin. Preferably the intron is the beta-globin IVS.

The octseq found in TK-promoter based plasmids is a non-coding sequence from octamer protein. It has no functional purpose in the plasmid, but was needed for creating suitable restriction sites for the preparation of the final plasmids.

The NEF, REV or TAT genes to be inserted into pBN can be obtained from several commercial sources such as the plasmid pKP59, which is available from the AIDS Reagent Project MHC repositary. Said genes are

well known and have been fully sequenced (Wain-Hobson, et al. Cell 40:9-17, 1985). Of course it is also possible to insert a sequence encoding only an immunologically active fragment of said HIV proteins.

The NEF, REV or TAT genes or their fragments are first inserted into appropriate shuttle vectors. These vectors can either include or not include the MO region. Two shuttle vectors are illustrated in the examples: pNp177, which does not include the MO, and pUE83, which includes the MO. Of course it is possible to use other shuttle vectors too. Both the shuttle vectors and the resulting vectors of the invention are preferably multiplied in Escherichia coli. Examples of the resulting pBN-NEF, pBN-REV or pBN-TAT vectors of the present invention are set forth in Figures 2, 3 and 4. The vectors of the invention are stable and self-replicating in a large copy number. Upon transfection into a eukaryotic host cell, the vector (plasmid) will multiply and produce 100 - 1000 fold amount of new plasmids, each capable of expressing the HIV protein in demand.

The host cell claimed in the present invention can be either a eukaryotic cell transfected by the vector or a prokaryotic cell transformed by the vector. The eukaryotic cell is preferably a mammalian cell and the prokaryotic cell is preferably a bacterial cell, especially E. coli.

The expression of HIV NEF, REV and TAT of the resulting plasmid vectors of the present invention was tested both in transfected COS-7 cells and in mice immunized with said plasmids. A high expression of the HIV proteins could be demonstrated in the COS-7 cells and the immunized mice showed a remarkable humoral and cell mediated (CTL) immune response. These results indicate that the vectors of the invention have a potential use as effective vaccines against HIV.

The present invention is further illustrated in the following examples. The examples describe in detail some embodiments of the invention, but they should not be interpreted to restrict the invention, which is defined by the attached claims.

Example 1

Cloning of HIV-1 genes REV and TAT into self-replicating pBNsr-α and pBNtk plasmids

Production of pBNtkREV and pBNsraTAT

Phase 1:

The HIV-1 REV and TAT genes from isolate BRU also called LAI (Wain-Hobson et al. Cell 40:9-17, 1985) were amplified from the pcREV and pcTAT vectors (Arya et al. Science 229:69-73, 1985) using Dynazyme Taq DNA polymerase (Finnzymes, Finland) and the following primers that have restriction enzyme sites for enzymes XhoI and XbaI:

For REV:
5′-TTTTTCTAGAACCATGGCAGGAAGAAGCGGA-3′
5′-TTTTCTCGAGCTATTCTTTAGTTCCTGG-3′

For TAT:
5′-TTTTTCTAGAACCATGGAGCCAGTAGATCCT-3′
5′-TTTTCTCGAGCTAATCGAACGGATCTGC-3′

The amplified genes and pUE83 shuttle vector (Figure 1) were digested at +37 °C with XbaI and XhoI (New England BioLabs, USA) overnight in order to get compatible ends. The digested DNA-fragments were analyzed on 1.5 % agarose gels, and further purified using Band Prep Kit (Pharmacia Biotech, Sweden). Each gene was ligated into the vector separately using T4 DNA ligase (New England BioLabs, USA) in an overnight incubation at +16 °C. The ligation products were transformed into One Shot Kit (Invitrogen, The Netherlands) competent E. coli cells, which were plated on LB-plates containing kanamycin for selection. Minipreps were prepared from the growing clones, and the presence of cloned genes was analyzed by digestion with XhoI and XbaI. The presence of the cloned genes was also confirmed by PCR from miniprep preparation using the above mentioned primers. Clones containing the right gene were mass cultivated and plasmids were purified using Megaprep columns (Qiagen, Germany).

Phase 2:

A DNA fragment containing BPVori, RSV LTR promoter, REV- or TAT-gene and b-globin IVS poly(A) was digested from the shuttle vector by HindIII (New England BioLabs, USA), and purified using 1 % agarose gel and Band Prep Kit. Ligation to HindIII digested and dephosporylated (alkaline phosphatase, CIP, Promega, USA) pBNsrα or pBNtk, transformation of cells,

verification of the presence of cloned gene and purification of the plasmid were done as in phase 1.

The resulting plasmids are called pBNtkREV and pBNsrαTAT and are set forth in Figures 2 and 3.

Example 2

Cloning of HIV-1 NEF into self-replicating pBNsrα plasmid

Production of pBNsrαNEF

Phase 1:

The HIV-1 NEF gene was obtained from a plasmid pcNEF vector, which contained the LAI isolate NEF gene inserted into a pcTAT vector lacking the TAT gene. The NEF gene used for further cloning was achieved as a 1.3 kb fragment by Spe I and Hind III digestion from pcNEF. To eliminate the reformation of the Hind III site on ligation, after Hind III digestion the fragment was treated with Klenow enzyme and a mix of dATP, dCTP, dGTP nucleotides after which the Spe I digestion was performed. The fragments obtained were separated by electrophoresis on a 1% agarose gel alongside standard size markers. Bands of correct size were cut out and the DNA recovered using the Sephaglas Bandprep Kit (Pharmacia Biotech), following the manufacturer's protocol.

The shuttle vector pNP177 of Figure 1B was first digested with Xho I, then treated with Klenow enzyme and dNTP mix, and, finally, digested with Xba I. The vector was also treated with calf intestinal alkaline phosphatase (CIP).

The fragment containing the NEF gene was ligated with cleaved pNP177 vector by using T4 ligase in +14°C overnight. One Shot competent E. coli kit (Invitrogen) was used for transformation. Positive clones were identified by using restriction enzyme digestions and electrophoresis. Plasmid DNA was further amplified in E. coli and purified in a large scale with Qiagen columns. The resulting final plasmid was called pNP177cHIVNEF.

Phase 2:

The shuttle vector pNP177 is designed to have only two Hind III sites between which an insert can be cloned. A Hind III digest of the plasmid thus gives a fragment which can be cloned further. The Hind III fragment of pNP177cHIVNEF was cloned into pBNsrα. The vector was digested with Hind III and treated with CIP. The same methods of band separation, ligation, transformation were used as in the first phase and correct orientation of the

insert was confirmed by restriction analysis. The final plasmid was called pBNsrαNEF and is shown i Figure 4.

Example 3

Demonstration of expression of HIV-Nef in vitro

3A.Transfections

To test the expression of the pBN-constructs of examples 1 and 2, they were transfected by electroporation into COS-7 cells. 10 μg of pBNβ-Gal as a control, and 10 μg of pBN-NEF cotransfected with 1μg pCMVβ-Gal, were electroporated each into three million cells. Salmon sperm carrier DNA was used. The electroporation was made at 960 mF capacitance and 260 V voltage. Protein concentration and β-gal activity measurements were made to control the efficiency of transfection and to calibrate the amount of the lysates in Western blot assay.

3B. Immunohistochemistry and Western blotting

The harvested cells transfected with the pBN-constructs were lysed for use in Western blotting. After lysis, protein samples were boiled in sample buffer and run in a 12% SDS polyacrylamide gel, then transferred onto a 2 μm nitrocellulose filter which was blocked with a solution of 5% milk in TBS. As a primary antibody a mixture of mouse anti-NEF monoclonals (Ovod V. et al. AIDS 6:25-34, 1992) diluted to 1:1000 each was used. The secondary antibody was a biotinylated anti-mouse in a 1:500 dilution.

After transfection to COS-7 cells, the vectors produced a strong transient HIV regulatory protein expression, as detected by Western blotting of the lysed cells at 72 hours. The results obtained with pBNsrαNEF are shown in Figure 5. In long-term cultures of the transfected cells, NEF expression sustained up to 7 weeks in the cells transfected with the self-replicating pBN vector.

The NEF-transfected cells were also used to prepare cytospin preparates and they were stained with haematoxylin and a monoclonal antibody against NEF followed by a secondary biotinylated anti- mouse were used in immunohistochemistry as described in Ovod et al. supra. The cytospin slides indicated expression as positive staining was seen in a large number of cells as granules occupying the cell cytoplasm. A portion of the NEF expressing cells showed morphological signs of cell destruction, indicating apoptosis. Still the level of expression was high though the condition of the cells was getting worse.

Example 4.

Demonstration of immunogenicity of pBNsrα and pBNtk vector expression of HIV-Nef, HIV-Tat or HIV-Rev in vivo

4A. Gene Gun

DNA was precipitated onto 1 μm gold particles using spermidine and $CaCl_2$ following the procedure in the Helios Gene Gun Instruction Manual (Bio-Rad Laboratories). Cartridges were made to carry 0.5 mg gold and 1 μg DNA each. The amount of DNA was controlled spectophotometrically as instructed in the manual. Inoculations were performed using the Helios Gene Gun System (Bio-Rad Laboratories). Helium discharge pressure for DNA delivery was set to 300 psi. In our optimization of the bombardment conditions we found 300 psi to be sufficient to propel the gold particles into the dermis.

4B. Immunizations

Female 6-8 week-old balb/c mice were used. Before immunizations the mice were anesthetized and the abdominal fur was removed.

Inoculations on the abdominal skin of 8 mice were done on days 1, 2, 3, 10, 11 and 12 using the gene gun described above and following the instruction of the manufacturer. A total of 6 μg of pBN-NEF was administered per mouse. Four mice from both groups were sacrificed two weeks post last immunization and the remaining four mice four weeks post last immunization. Serum samples for Western blotting were taken and splenocytes harvested for a CTL assay. All eight mice immunized with the pBN-NEF- vector, showed an antibody response at 2 weeks and 4 weeks (Figure 6). The intensity of the reaction in the Western blotting varied.

4C. Measurement of cytotoxic T-cell activity in the immunized mice

4C1. Stimulation of effector cells

Spleens were removed aseptically from the immunized mice two (16 mice) and four weeks (16 mice) after immunization. They were disrupted in Hanks, filtered through gauze and the erythrocytes were removed. Cells were then suspended 5 x 106 / ml in culture medium: RPMI 1640 medium containing 10 % fettle calf serum (FCS; GibcoBRL), 1 % glutamin, 100 U of penicillin per ml, 100 (g of streptomycin per ml and 5 x 10-5 M 2-mercaptoethanol. The responding cells (5 x 106) were co-cultured in 25 ml cell culture flask in 5 ml of culture medium with 4 x 106 antigen presenting cells (APCs; see below) for five days. 10 U / ml of recombinant interleukin - 2 (rIL-2)

was added at the first day to the cells (Hiserodt J. et al. J. Immunol. Jul, 135(1):53-59, 1985; Lagranderie M. et al. J. Virol. Mar, 71(3):2303-2309, 1997; Tsuji T. et al. Immunology Jan. 90(1):1-6, 1997; Vahlsing H. L. et al. Journal of Immunological Methods 175:11-22, 1994; Varkila K. et al. Acta path. Micorbiol. Immunol. Scand. Sect. C 95:141-148, 1987)

4C2. Antigen presenting cells

Syngeneic P815 mastocytoma (H-2d) cells were infected with modified vaccinia virus Ankara (MVA) engineered to express the HIV-1 LAI NEF gene (MVA-HIVNEF). MVA is a highly attenuated replication-deficient vaccinia virus, which can serve as an efficient vector for expression of heterologous genes providing an exceptionally high level of biological safety (Sutter G. et al. J. Virol. Jul, 68(7):4109-4116, 1994; Sutter et al. Vaccine 12(11):1032-1039, 1994; Drexler I. et al. J. Gen. Virol. 79:347-352, 1998; Sutter G. et al. Proc. Natl. Acad. Sci. USA 89:10847-10851, 1992). Infections with MVA-HIVNEF were performed at a multiplicity of infection (MOI) of 5 in 24 - well plates (1 x 10^6 cells per well). After 1h virus absorption at +37°C, the cells were incubated for 15 h in +37°C (Carmichael A. et al. Journal of Virology 70:8468-8476, 1996). After infection the cells were washed twice with PBS (phosphate buffered saline) containing 10 % FCS and suspended in this solution 5 x 10^6 cells/ml. Cells were then γ-irradiated at 5000 rad and washed with culture medium before adding to responder cells.

4C3. Cytotoxicity assays

CTL activity was tested by the ^{51}Cr- release assay (Hiserodt J. et al. J. Immunol. Jul, 135(1):53-59, 1985; Lagranderie M. et al. J. Virol. Mar, 71(3):2303-2309, 1997; Varkila K. et al. Acta path. Micorbiol. Immunol. Scand. Sect. C 95:141-148, 1987; van Baalen C. et al. AIDS 7:781-786, 1993). Briefly, 2 x 10^6 P-815 cells were infected with MVA-HIVNEF as described above for antigen presenting cells. After infection the cells were washed once in serum free culture medium. Target cells then were suspended in 200 µl of serum free culture medium and 100 µCi of ^{51}Cr (Amersham) / 1 x 10^6 cells was added for 1 h at 37°C. Target cells were then washed four times in medium and suspended in concentration 5 x 10^4 / ml. The stimulated effector cells were washed once in culture medium before adding to the target cells. Target cells were plated in u-bottom 96- well plate 100 µl (5 x 10^3) per well and effector cells were added in triplicates in 100 µl at effector: target ratios 50, 25 and 12.5. For spontaneous release, target cells were plated in six wells with 100 µl

of culture medium and for maximum release in six wells with 2.5 % Triton-X-100. The plates were spun briefly, incubated for 4 hours in 37°C and the supernatants were counted in a gamma-counter. The percent specific lysis of target cells was calculated as (test ^{51}Cr release - spontaneous release)/(maximum release - spontaneous release) x 100. The percent specific lysis ≥ 6 % was considered to be positive.

An example showing CTC activity in 6 of the 8 mice immunized with pBNsrαNEF is shown in figure 7.

D. Humoral immune response in immunized mice

To test the occurrence of antibodies against HIV-1 NEF in immunized mice sera NEF protein was electrophoresed on PAGE, transferred to nitrocellulose filters and the antibody reactivity was detected as described above.

Summary of the results

The results of the transfection and immunization tests are summarized in Table 1. The immune response in the immunized mice was assessed by immunoblotting (WB) for humoral and by cytotoxic t-lymphocyte (CTL) assay for the cell mediated immunity. As seen in the table, all eight mice immunized with the NEF expressing vector showed both humoral and cell mediated immune response.

Table 1. Demonstration by immunoblotting (Western blotting, WB) or by immunohistochemistry of the expression of the HIV-1 NEF, TAT and REV proteins in COS-7 cells transfected with said vectors and demonstration of induction of humoral and cell mediated immune response in mice immunized with one of the vectors, pBNsrαNEF

	Transfection		Immunization	
	WB	Immunohisto-chemistry	WB	CTL
pBNsrαTAT	ND	++	6/8	6/8
pBNtkREV	+	++	7/8	7/8
pBNsrαNEF	++	ND	6/8	6/8

In this study we demonstrate that DNA immunization using a self-replicating expression vector as described can induce a clearly detectable CTL

response in mice. In addition a humoral immune response was achieved. In view of the above results it is feasible to assume that the pBN-NEF, pBN-REV and pBN-TAT plasmids do express NEF, REV and TAT in vivo in an amount sufficient to induce both the humoral and the cell-mediated immune response necessary for preventing or treating HIV.

Claims

1. A self-replicating recombinant vector comprising papilloma virus nucleotide sequences consisting essentially of

(i) a papilloma E1 gene and E2 gene,

(ii) a minimal origin of replication of a papilloma virus

(iii) a minichromosomal maintenance element of a papilloma virus, and

a heterologous nucleotide sequence encoding the HIV regulatory protein NEF, REV or TAT or an immunologically active fragment thereof.

2. A self-replicating vector of claim 1 wherein the papilloma virus is bovine papilloma virus (BPV).

3. A self-replicating vector of claim 1 or 2 wherein the heterologous nucleotide sequence encodes the HIV-1 NEF protein.

4. A self-replicating vector of any of the preceding claims wherein E1 is under the control of the srα promotor or the thymidine kinase promotor.

5. A self-replicating vector of claim 4 which is pBNtkREV, pBNsrαTAT or pBNsrαNEF as shown i Figure 2, 3 or 4.

6. A vaccine for DNA immunization against HIV comprising the self-replicating vector of any of claims 1 - 5.

7. Method for preparing a self-replicating recombinant vector of any of claims 1 - 5, said method comprising

A) inserting a heterologous nucleotide sequence encoding the HIV regulatory protein NEF, REV or TAT or an immunologically active fragment thereof into a vector comprising papilloma virus nucleotide sequences consisting essentially of

(i) a papilloma E1 gene and E2 gene,

(ii) a minimal origin of replication of a papilloma virus, and

(iii) a minichromosomal maintenance element of a papilloma virus, and

B) transforming a host cell with the resulting self-replicating recombinant vector,

C) culturing the host cell, and

D) recovering said vector.

8. The method of claim 7 wherein the host cell is an E. coli cell.

9. Use of the self-replicating vector of any of claims 1 - 5 for the manufacture of a DNA immunization vaccine against HIV.

10. Method of treating or preventing HIV comprising administering to a person in need thereof an effective amount of the self-replicating vector of any of claims 1 - 5, and expressing the NEF, REV or TAT protein or an immunologically active fragment thereof in said person.

11. A host cell comprising the self-replicating vector of any of claims 1 - 5.

12. The host cell of claim 11, which is a bacterial cell or a mammalian cell.

Abstract

A nucleotide sequence encoding the HIV regulatory protein NEF, REV or TAT or an immunologically active fragment thereof is inserted into a vector comprising papilloma virus nucleotide sequences necessary and sufficient for long-term persistence. The resulting vectors are self-replicating and have a high copy number. They express the HIV genes in high amounts for a long period of time. The vectors elicit both a humoral and cell-mediated immune response and are therefore potential DNA immunization vaccines against HIV. The invention is directed to said vectors and vaccines and to a method for preparing the vectors. The invention is further directed to a host cell comprising the vector, to the use of the vector in the manufacture of a vaccine and to a method of preventing or treating HIV.

Figure 1A

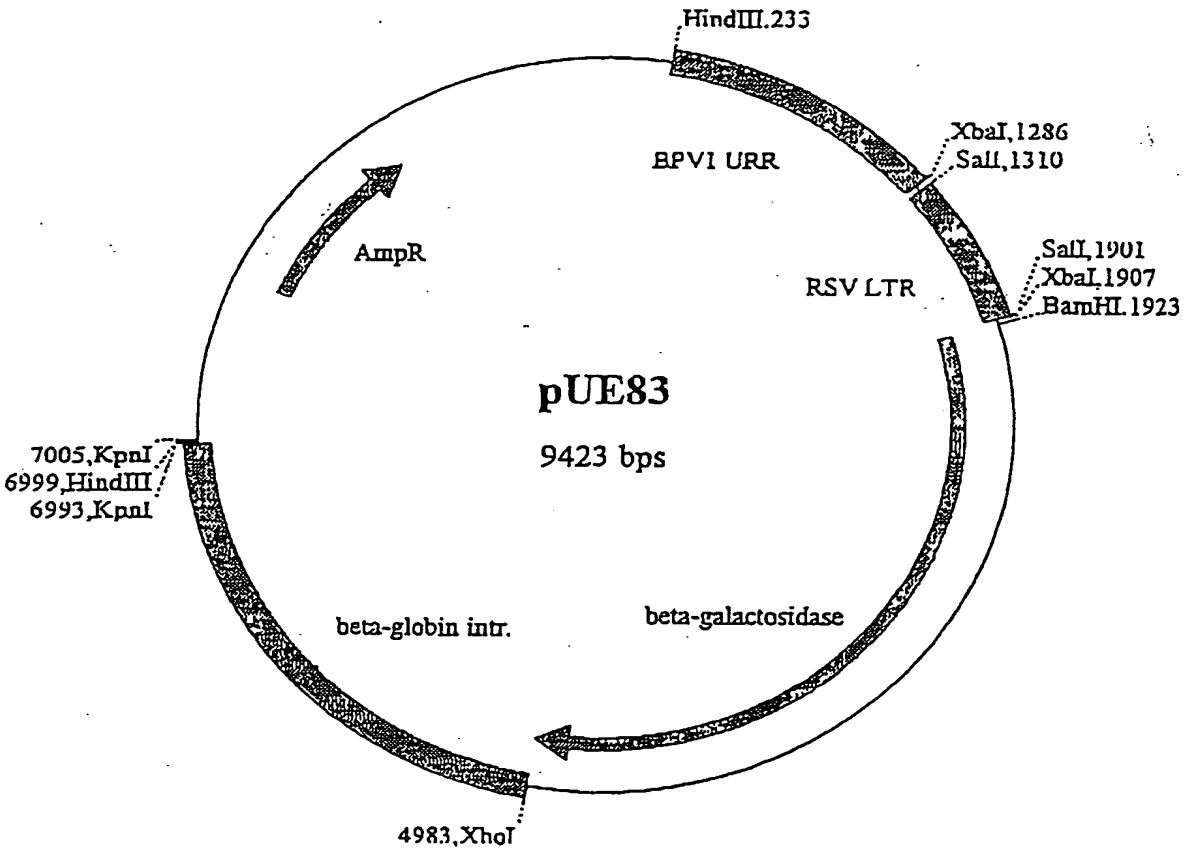

HindIII.233
XbaI,1286
SalI,1310
BPVI URR
AmpR
SalI,1901
XbaI,1907
BamHI.1923
RSV LTR
pUE83
9423 bps
7005,KpnI
6999,HindIII
6993,KpnI
beta-globin intr.
beta-galactosidase
4983,XhoI

Figure 1B

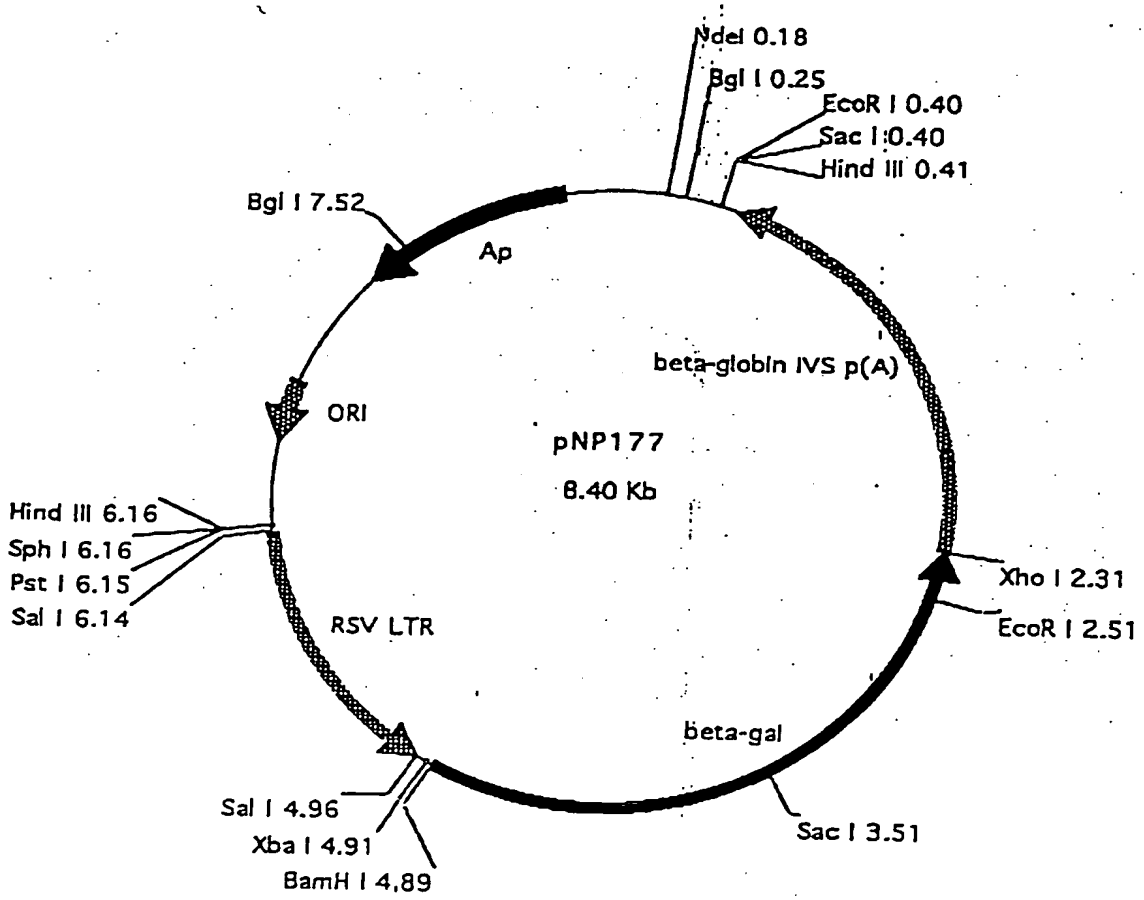

NdeI 0.18
Bgl I 0.25
EcoR I 0.40
Sac I 0.40
Hind III 0.41
Bgl I 7.52
Ap
beta-globin IVS p(A)
ORI
pNP177
8.40 Kb
Hind III 6.16
Sph I 6.16
Pst I 6.15
Sal I 6.14
RSV LTR
Xho I 2.31
EcoR I 2.51
beta-gal
Sal I 4.96
Xba I 4.91
BamH I 4.89
Sac I 3.51

Figure 2

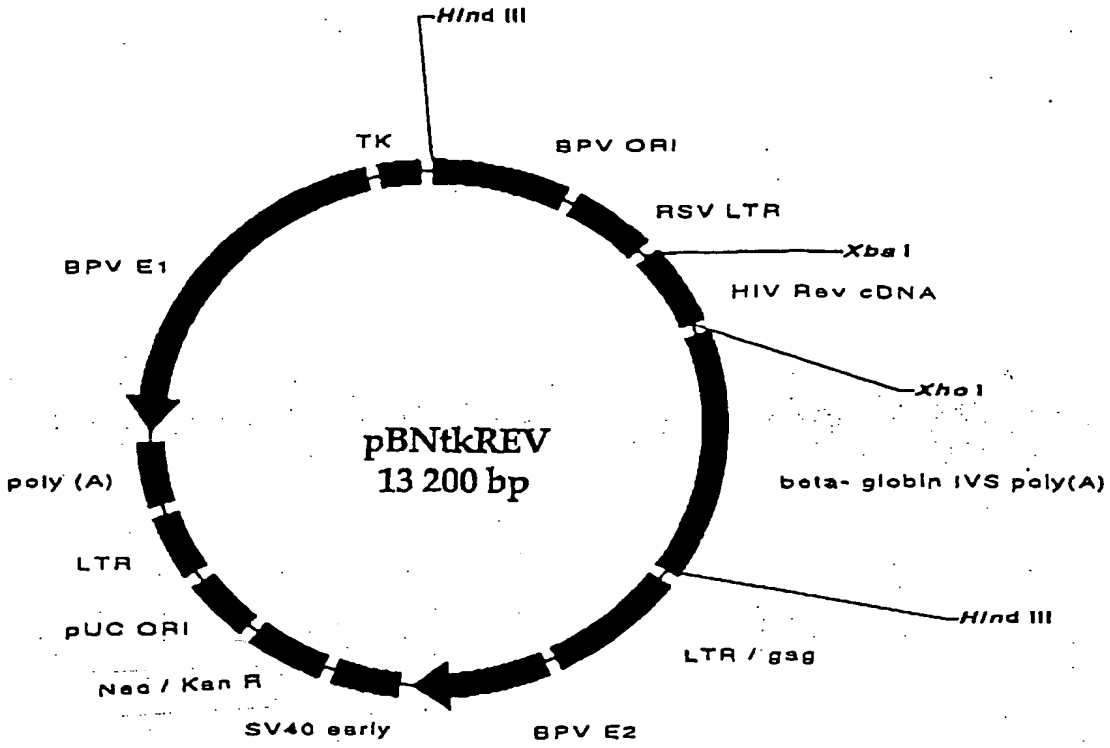

Hind III
TK
BPV ORI
RSV LTR
Xba I
BPV E1
HIV Rev cDNA
Xho I
pBNtkREV
13 200 bp
poly (A)
beta- globin IVS poly(A)
LTR
Hind III
pUC ORI
LTR / gag
Neo / Kan R
SV40 early
BPV E2

Figure 3

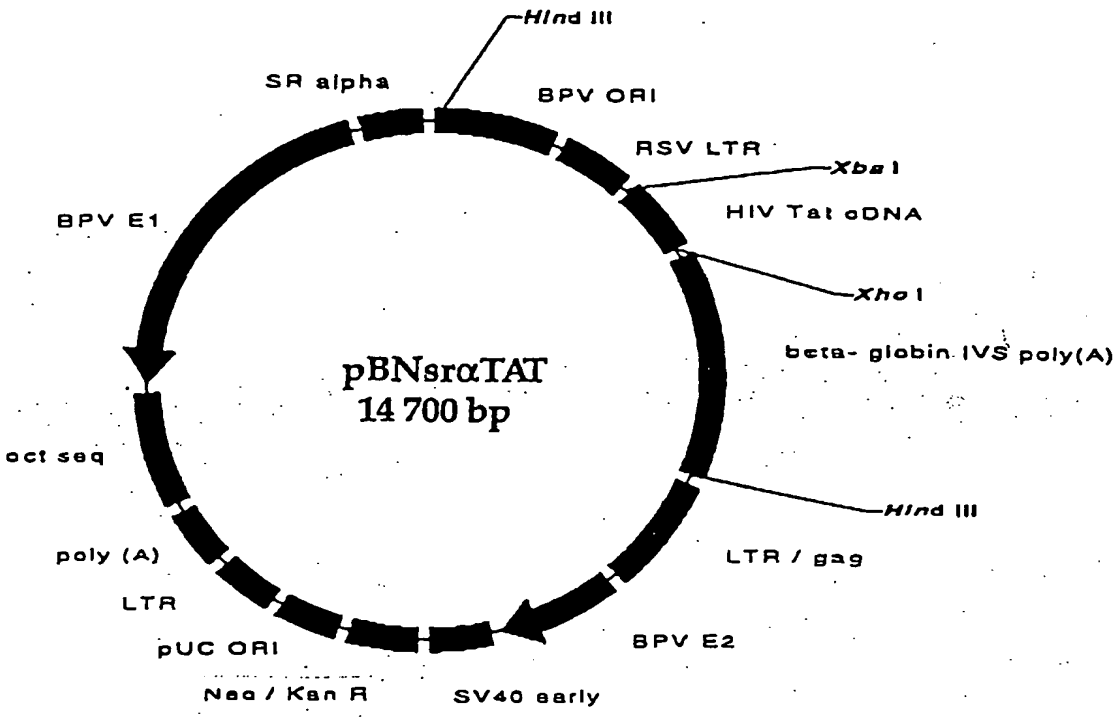

Hind III
SR alpha
BPV ORI
RSV LTR
Xba I
HIV Tat cDNA
BPV E1
Xho I
beta- globin IVS poly(A)
pBNsrαTAT
14 700 bp
oct seq
Hind III
poly (A)
LTR / gag
LTR
pUC ORI
BPV E2
Neo / Kan R
SV40 early

Figure 4

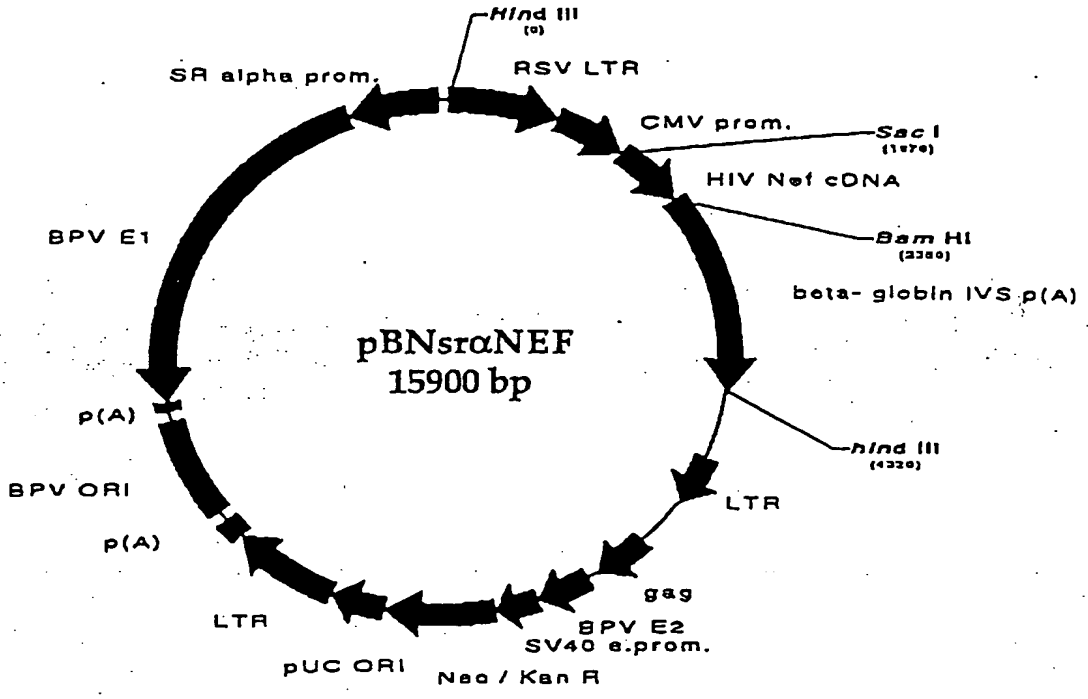

Hind III
(0)
SR alpha prom.
RSV LTR
CMV prom.
Sac I
(1070)
HIV Nef cDNA
Bam HI
(2380)
beta- globin IVS p(A)
BPV E1
pBNsrαNEF
15900 bp
p(A)
hind III
(4320)
BPV ORI
LTR
p(A)
gag
LTR
BPV E2
SV40 e.prom.
pUC ORI
Neo / Kan R

Figure 5

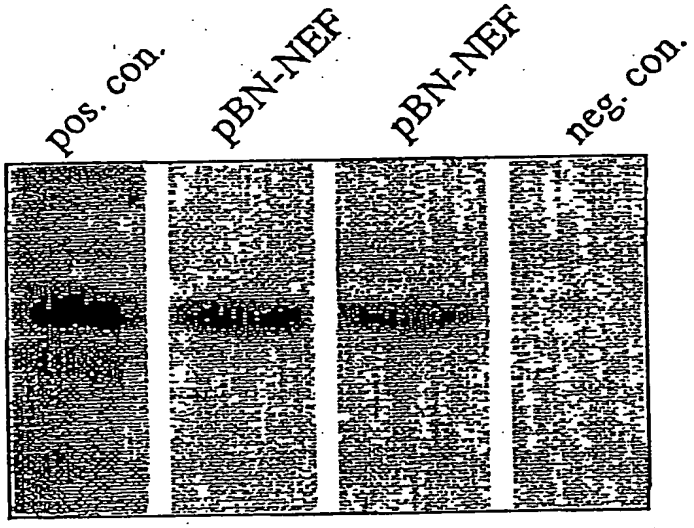


Figure 6

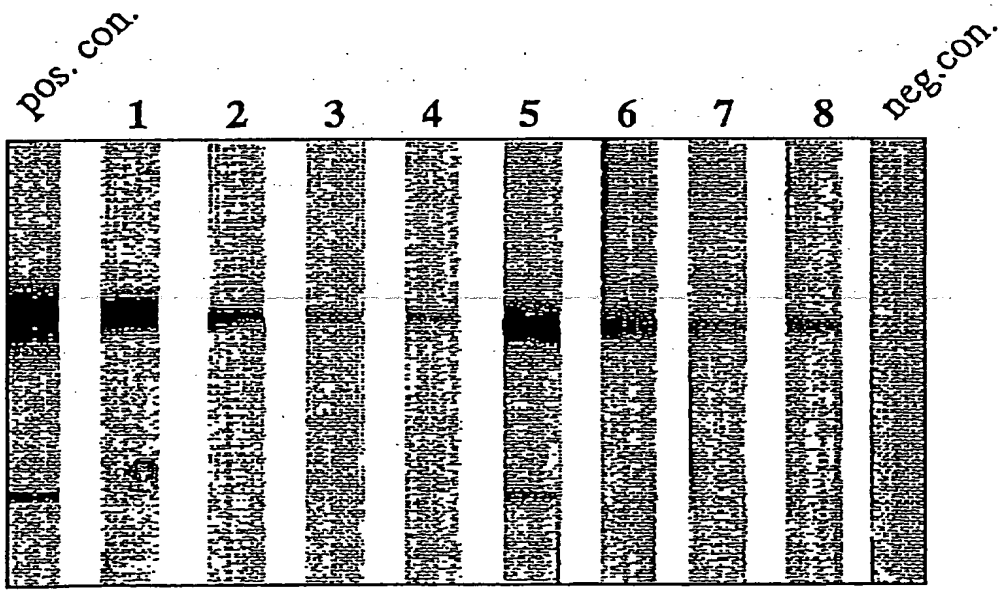
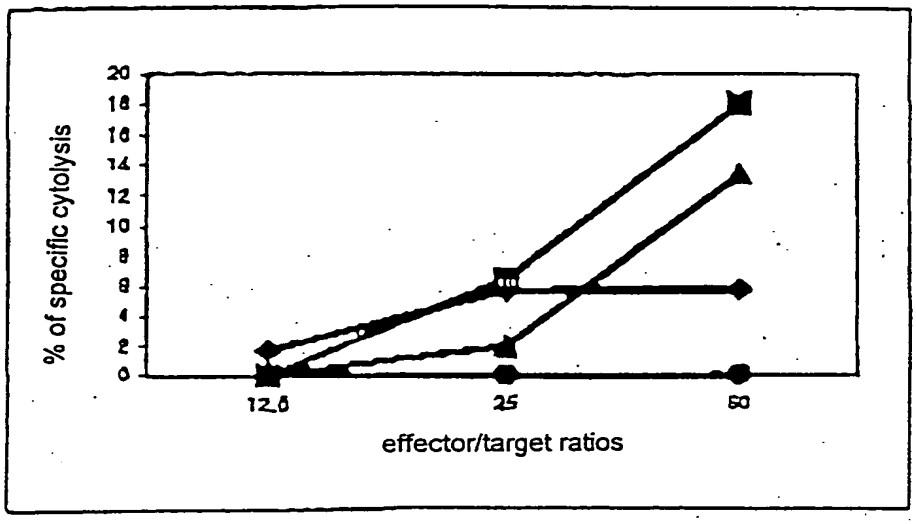
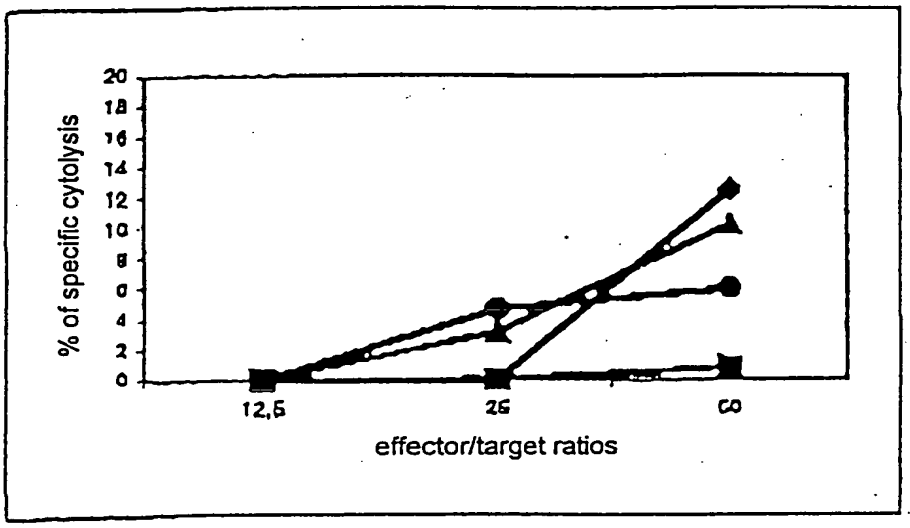


Figure 7

A


% of specific cytolysis
20
18
16
14
12
10
8
6
4
2
0
12,5
25
50
effector/target ratios

B


% of specific cytolysis
20
18
16
14
12
10
8
6
4
2
0
12,5
25
∞
effector/target ratios